Jurnal Perlindungan Tanaman Indonesia, Vol .13, No.1 , 2007 : 63-72.

CHARACTERIZATION OF A BACULOVIRUS OF *SPODOPTERA LITURA* (LEPIDOPTERA: NOTUIDAE) ISOLATED FROM YOGYAKARTA

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ABSTRACT

A baculovirus has been isolated from cadaver of larvae of Spodoptera litura, *a Noctuidae of agricultural importance due to it wide range hosts and the damage to their respective hosts. Phase contrast light microscopy observation from infected larvae, showed that the fat body, hemocyte cells, and cells surrounding the trachea or tracheolus were the most tissue invaded by polyhedra. Transmission electron microscopy analysis of the occlusion body purified from diseased larva showed that the baculovirus envelope containing multiple nucleocapsid. Digestion of viral DNA with three restriction enzymes showed that the genom pattern of baculovirus isolated from Bantul were close to SpliNPV isolated from Japan and those of* Spodoptera littoralis *and quite distinct from those isolated from Southeast Asia region. Bioassay test performed on first to fifth instar larvae showed that the virus effectively control the young larvae, but showed some level of resistance against older larvae of* Spodoptera litura

Keywords : *Characterization; baculovirus;* Spodoptera litura*; Yogyakarta*

INTISARI

Ditemukan patogen Baculovirus dari larva *Spodoptera litura* pada pertanaman bawang merah di Bantul. Pengamatan dengan menggunakan mikroskup fase kontras pada beberapa jaringan larva yang terinfeksi virus, menunjukkan bahwa bagian lemak, sel darah, dan sel di sekitar trakhea dan trakheolus merupakan tempat dimana banyak ditemukan polihedra. Hasil pengamatan dengan mikroskup elektron transmisi menunjukkan bahwa virus ini merupakan multiple nucleocapsid. Digesti DNA virus menunjukkan bahwa pola genomnya dekat dengan virus yang diisolasi dari *S. exugua* di Jepang dan *S. littoralis* akan tetapi berbeda dengan isolat Asia Tenggara. Hasil uji bioassay menunjukkan bahwa virus ini cukup efektif dalam mengendalikan larva muda, tetapi menunjukkan adanya resistensi pada larva yang telah tua

Kata kunci: Karakterisasi, baculovirus, *Spodoptera litura*, Yogyakarta

INTRODUCTION

Baculoviruses are enveloped viruses that have double stranded, circular DNA genomes ranging in size from 90 to 180 kbp (Volkman *et al.*, 1995). This group of invertebrate specific viruses cause infection in more than 600 spcies of insect and has been used as microbial pesticides to control the population of insect pests of agriculture, forestry, and pasture (Moscardi, 1999). Baculoviruses are recognized as one of the most important entomopathogens being developed as microbial insecticide due to their safety for non-target organism, environmentally persistent, and showed high virulence against target insect pest. It is clear that baculovirus have a substantial impact on the natural population dynamics of insects (Fuxa and Tanada, 1987). Recently, baculoviruses also play an important role as vectors for the expression of exogeneous protein of interest for research and pharmaceutical purposes (Luckow, 1995).

Baculovirus infection are characterized by a biphasic replication cycle, during which two type of virion, budded virus (BV) and occlusion-derived virus (ODV), are produced. The BV initiated secondary infection and spread the infection throughout the host tissues; the ODV packaged within the occlusions body (OB) is released from the diseased insect and transmit the virus to the host population by initiating primary infection in the midgut epithelial cells (Frederici, 1997). The family Baculoviridae compromises the two genera Granulovirus (GV) and Nucleopolyhedrovirus (NPV), although recent genome sequence analysis have indicated that this family should include four genera. Furthermore, lepidopteran specific NPVs has been divided into two groups based on single gene polygenies, which was corroborated by phylogenies inferred from whole genome sequences (Herniou *et al.*, 2003). The major protein of the inclusion body, the polyhedrin has been characterized in many different virus and was shown to be highly conserved.

Spodoptera litura is an extremely polyphagus insect pest that is widely distributed in tropical, subtropical and temperate regions of the world. It host icluding more than 200 plant species, including economically important crops, such as cabbage, onion, tabaco, and chilli (Smith, 1987). An Spli NPV isolate from Japan now is under process for commercialization (Kunimi, personal communication), and another NPV has been commercially available for controlling cabbage army worm, *Mamestra brassicae* (Kunimi, 1998). In Indonesia *S. litura* also becoming a big problem in important crops and causes serious damage to plant such as cabbage, chili, tomato, soybean and several estate crops, e.g. cotton and tobacco. Controlling this insect becoming more difficult since the species also developed resistance to many chemical pesticides (Takatsuka and Kunimi, 2002).

In this paper, we describe the isolation, purifcation and partial characterization of the baculovirus isolated from cadaver of *Spodoptera litura* larvae recovered from onion plantation in Bantul,

Yogyakarta. This will hopefully increase the information of the currently limited knowledge of baculovirus ecology.

MATERIAL AND METHODS

Virus propagation. Spli NPV was recovered and isolated from cadaver of *S. litura* collected from onion plantation in Bantul, Yogyakarta. The virus was passed orally through third and fourth instar of *S. litura* larvae reared in wheat germ-based artificial diets for propagation. Infected larvae were processed further to obtain the OBs. The OBs then were purified by homogenization and differential centrifugation using high speed centrifuge (Kokusan, H9R) as described by Ishii *et al.* (2003). The concentration of the OBs was determined using a Thoma hemocytometer under a phase-contrast microscope (Olympus, CX 41, Tokyo, Japan). The purified OBs was the restored on-30°C until used for experiments.

Microscope observation. Infected larvae were disssected in phosphate buffer (pH 7.5) to keep the osmotic balance. In certain tissue, observation was performed by directly dissected infected larvae and mounted onto the slide glass deck and checked for the presence of polyhedra with phase contrast microscope. Fatbody cells, Malphigi tubules, hemocyte cells, epidermis, and trachea are the tissues where the polyhedra were propagated. The purified polyhedra were observed under a transmission electron microscope in Graduate School of Agriculture, Tokyo University of Agriculture and Technology as follows. Purified polyhedra were fixed in modified fixative (2.5% glutardehyde, 2% paraformaldehyde in 0.05 M, pH 7.2, cacodylate buffer + 0.001 M $CaCl_2$) for 2 h, post fixed in 1% osmium tetroxide in the same buffer for 1 h and then stained in *en bloc* with 0.5% aqueous uranyl acetate, dehydrated in acetone, embedded in Spurr low viscocity embedding medium. Bloc were sectioned in a Leica Ultracut N microtome, stained with 3% aquaeus uranyl acetat and lead citrate, and examined in a transmission electron microscope (H-7500, Hitachi, Tokyo, Japan) at 80 kV.

Viral DNA extraction and digestion with restriction enzyme. Polyhedra were treated with 50 mM Na_2CO_3 at room temperature for 30 min to release the virions. Viral DNA was extracted from purified OBs using a PUREGEN Tissue DNA Purification Kit (Qiagen, Tokyo, Japan). For the restriction enzyme treatment, approximately 1 µg viral DNA was digested with 10 U of restriction enzyme *Eco*RI, *BamH*Iand *Pst*I. DNA samples were then subjected to electrophoresis in a 0.8% agarose gel containing ethidium bromide following standard procedure (Sambrook, *et al.*, 1989) and photographed on UV transilluminator with DIANA II Chemiluminesence Detection System with Gene Profiler to estimate the molecular weight of DNA fragments.

Bioassay. First to fifth instar larvae were bioassayed using droplet feeding method based on Hughes and Wood, (1981) with some modification. Head capsule slippage was used to determine when the larvae

begin to molt. Neonate and newly molted larvae were allowed to feed on droplets of POB containing 10% sucrose and red food coloring. Control larvae at each stage were fed droplet of the same solution without OBs. After ingesting the solution marked by changing colour of the body, larvae were immidiately transferrred to cups containing fresh artificial diet. The volume of suspension ingested by first to second stage larvae was determined by fluorescent dye methods and by the gravimetric method for the rest of the stage according to Kunimi and Fuxa (1996). Four doses plus control were used for each instar and 30 larvae were used for each dose. Larvae were reared at 25°C with a 16-h photoperiod.

To measure mortality, larvae were observed daily until pupation. Tissue smears from dead larvae were prepared and examined for the presence of OBs under phase contrast microscopy Olympus, CX-41; Tokyo, Japan).

Data analysis. Mortality data were analyzed by probit analysis (Finney, 1978) using free computer programe SAKUMA PROBIT analysis according to Sakuma (1998).

RESULTS AND DISCUSSION

Viral production. Infected larvae were pooled and weighted for determining the capacity of virus production. From total 64.83 gr infected larvae processed for purification resulted in
8 ml virus suspension with PIB concentration as much as 3.125×10^{10} PIB/ml. This assay also showed that the optimum virus production will be obtained by assaying in fourth instar larvae. Using younger larvae would result in fast speed of infection which produced less polyhedra, and using older larvae might lead to low infection rate due to the "dilution effect". This aspect is an important factor for baculovirus production as it will be proceeded for making microbial insecticide.

Electron microscopy. Tranmission electron micrograph study revealed that the virus isolated from *S. litura* cadaver in Bantul, Yogyakarta was typical baculovirus occlusion bodies (Fig. 1), and showed that the occlusion bodies were of irregular shape and range from 1.5 to 4.5 μm in diameter. The occlusion bodies were occupied by several virions with multiple

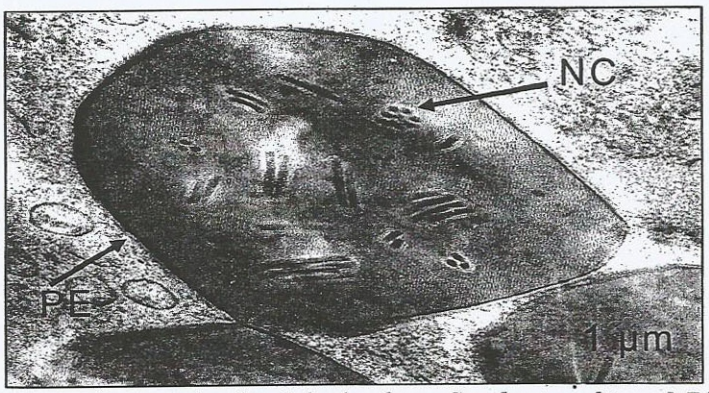

Figure 1. Electron micrograph of polyhedra from *Spodoptera litura* MNPV
PE : Polyhedron envelope; NC : Rod-like nucleocapsid

nucleocapsids packaged within a single viral envelope. From this finding we named the virus as SpliMNPV.

Infected tissue observation. The best time to observe the internal tissue and organ of the baculovirus-infected larvae is at the time when the larvae showed lethargic behaviour or stop feeding on the diet. The infected tissue will be in intact condition and the developed polyhedra still stayed within the nucleus of the infected cell. Microscopic observation showed that the polyhedra could be found in an abundant number in the cell surrounding trachea or tracheolus (Fig. 2.A), in the hemocyte cell (Fig. 2.B), and in the fatbody cells (Fig. 2.C). Polyhedra were

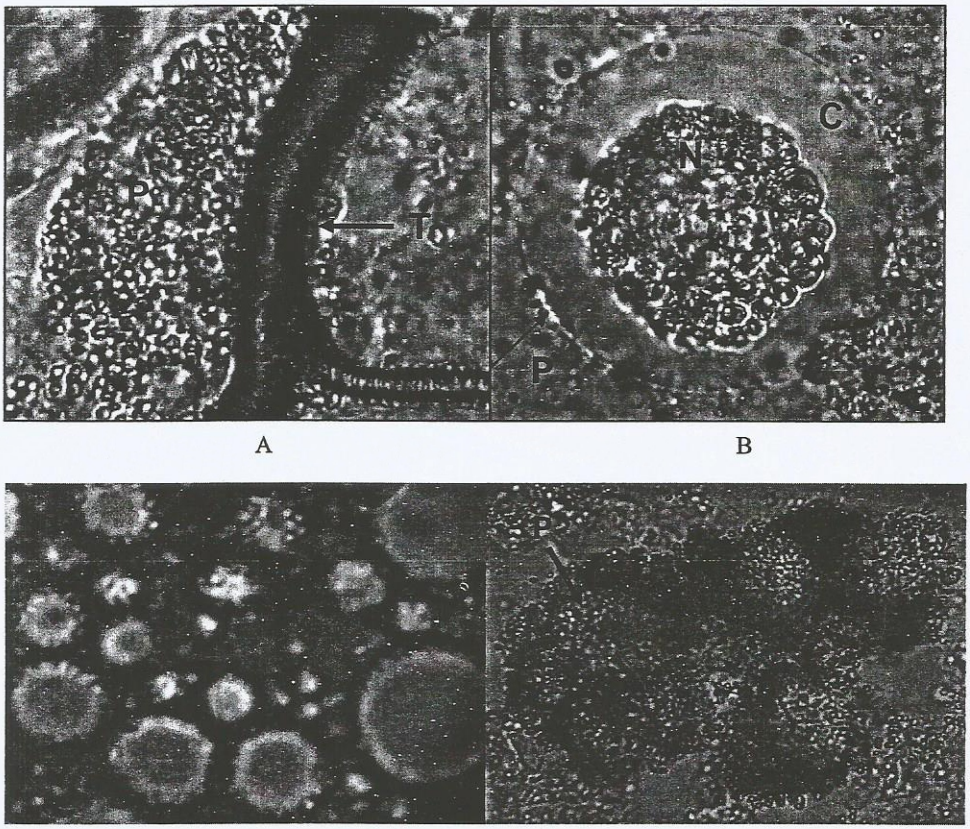

Figure 2. (A). Baculovirus infected-cell surround trachea
T: Tracheolus; P: infected cells full of polyhedra
(B). Baculovirus-infected hemocyte cell
P: Plasma membrane; N: Infected nucleus full of polyhedra;
C: Cytoplasm
(C). Normal fatbody cells
(D). Baculovirus-infected fatbody cells
P: Polyhedra within fatbody cells

not found in the malphigi and nerve system. Some insect pathogens sometimes showed tissue preference for propagation within their host. Entomopoxvirus and Granulovirus prefered to propagate in the fatbody (Tanada and Fuxa, 1987), and certain microsporidia isolated from *S. litura* showed tendency to propagated in nerve system (Iwano, personal communication). The qualitative amount of polyhedra observed from internal tissue and cells from baculovirus-infected larvae was represented in Table 1. SpliMNPV isolated from Bantul showed that the polyhedra were propagated in several tissues of the host larvae.

Restriction enzyme profile of SpliMNPV. Restriction enzyme analysis of the genome of SpliMNPV yielded in 22 *Eco*RI fragments, 11 *BamH*I fragments and 11 *Pst*I fragments (Fig. 3). The size of the restriction endonuclease fragments, estimated by the graphical method developed by Southern (1979) are shown in Table 2. Volkman *et al.*, (1995) stated that the baculoviruses genomes ranges in size from 90 to 180 kbp, and the genome size of the SpliNPV from Bantul was around 124 to 126 kbp. The genome profiles of this Bantul isolat SpliMNPV are similar to those of *Spodoptera littoralis* NPV isolated from Midle East and *Spodoptera litura* MNPV isolated from Japan area, but showed significant differences in genome pattern with those of SpliNPV isolated from Vietnam or Malaysia (Kunimi, unpublished data). It is tempting to collect more baculovirus from other region in Indonesia to study its phylogeny variation and its biological activity.

Table 1. Distribution of polyhedra within infected *Spodoptera litura* larvae

Tissue or cell observed	Qualitative number of polyhedra
Trachea/tracheolus	+++
Epidermis	++
Fatbody	++
Hemocytes	+++
Nerve	+
Midgut	++
Malphigi	-

Table 2. Restriction endonuclease fragment size of SpliMNPV DNA*

Fragment	*EcoR*I	*Pst*I	*BamH*I
A	18.4	20.2	26.5
B	14.8	18.2	22.3
C	11.2	16.0	20.2
D	9.1	16.2	19.3
E	9.0	15.3	10.0
F	7.0	15.0	7.2
G	6.4	11.2	6.2
H	6.2	7.2	5.2
I	5.9	4.0	4.2
J	5.2	2.0	2.2
K	4.3	1.5	1.5
L	4.2		
M	3.9		
N	3.5		
O	3.2		
P	2.9		
Q	2.3		
R	2.2		
S	1.8		
T	1.4		
U	1.3		
V	1.1		
Total	125.3	126.8	124.8

* Size of individual fragments in kilobase pairs

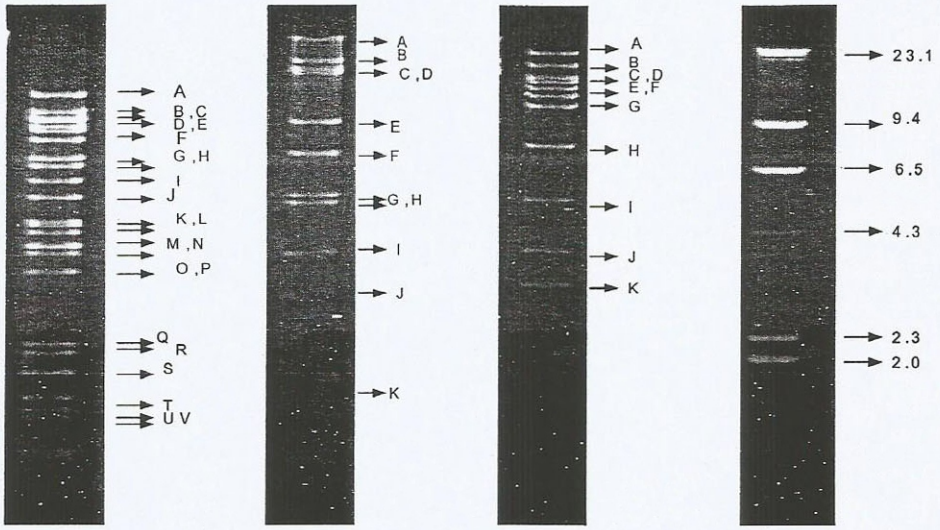

Figure 3. Restriction enzyme profile of SpliMNPV DNA digested with *EcoR*I, *BamH*I, and *Pst*I. *Hind*III is molecular marker on right end

Bioassay of SpliMNPV on *S. litura* larvae. Figure 4 summarizes mortality in various instar of *S. litura* larvae following ingestion of POB solution. The mortality data showed that SpliMNPV isolated from Bantul are highly effective in controlling young instar larvae. Inoculation with 10^5 per larvae resulting in 96.67; 76.67; and 56.67 percent for first, second and third instar larvae, respectively, but showed lower infection rate on the forth instar larvae, and no infection on the fifth instar larvae (data not shown).

The LD_{50} value (POB/larva) increased with the host stage at the time of viral treatment, ranged from 3.11 to 8.33 (Table 3). The slope of log dose-probit line were 0.36 – 0.94. Generally the susceptibility of lepidopteran insect to baculovirus decreases with the stage as explained by the "dilution effect": the decrease in susceptibility is related to an increase in larval weight as development proceeds (Briese, 1986). The mechanism and ecological cause of this phenomenon need to be adressed further, with regard to the effectiveness of the baculovirus as microbial insecticide. Huber and Hughes (1984) hypothesized that at lower concentration, most baculovirus in individual test insects begin with infection by a single or few virions, which require more time to multiply and kill the insect.

Therefore, particularly for baculovirus-based insecticide, precise time for application, e.g. coincident with larval hatch or young larval application, is required to obtain best result. Ecological aspect of the baculovirus as natural control agent need to be addressed further.

ACKNOWLEDGEMENTS

Author thanks the anonimous reviewer for useful comments on this manuscript. This research was partially funded by Hibah Bersaing from DIKTI and Monbukagakusho Scholarship for Ms. Nuri Yusmarlita.

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